REMARKS

Claims 1-20 are pending in the present patent application. Claims 1-20 have been finally rejected in an Office Action dated December 30, 2005.

On March 6, 2006, Ben Warner, Bruce Cottrell, and Sam Borkowsky conducted an interview with Examiner Davis and Examiner Ceperley at the U.S. Patent Office. Applicant wishes to thank the Examiners for the courtesies extended during the interview. During the interview, Examiner Ceperley suggested amending claim 1, step (a) by including the phrase being detectable by X-ray fluorescence after "binder" for clarity. Applicant agreed to the change. An interview summary was mailed to Applicant on March 17, which stated that the phrase would be entered after final. Not included in the interview summary were some of the other discussion that took place during the interview, such as when Applicant mentioned that a company had been formed for implementing the invention, and that an R&D award for the invention was received. During the interview, the filing of an Appeal was also suggested.

On April 21, 2006, Applicant filed a Notice of Appeal (with a one-month extension of time) on April 21, 2006. Applicant is now filing a Request for Continued Examination (RCE). Applicant is submitting this paper with the RCE. Attached to the paper is Evidence that has not yet been entered into the record. Please consider the following remarks and the Evidence.

According to the Office Action of December 30, 2005, claims 1-8, 10-18, and 20 were finally rejected as being obvious under 35 U.S.C. §103(a) over PCT Application Number WO 90/15070) to Pirrung et al. (hereafter referred to as Pirrung) in view of U.S. Patent 6,041,095 to Boris Yokhin (hereafter referred to as Yokhin).

Pirrung teaches a method for determining chemical binding using photo-activated receptor arrays and binders. According to Pirrung, an array is activated before exposing it to binder(s). An array is activated first, and the activated array is exposed to binders and then analyzed to determine whether or not any binder is bound to a member (a receptor) of the activated array. Pirrung discloses several ways to analyze whether or not this binding has occurred. These ways include using autoradiography, ultraviolet light, and visible light. Pirrung does not disclose, nor suggest, x-ray

fluorescence detection to detect chemical binding between binders and members of a receptor array. According to the Office Action of December 30, 2005, the method of Pirrung is silent with respect to using x-ray fluorescence to analyze members of an array for chemical binding.

Yokhin teaches an apparatus for performing x-ray fluorescence. According to the Office Action of December 30, 2005, Yokhin mentions that x-ray fluorescence is a well-known technique for determining elemental compositions. According to the Office Action, the Examiner is providing the motivation for combining Yokhin with Pirrung because of Yokhin's disclosure that x-ray fluorescence is a well-known technique for determining elemental compositions. The Examiner also states that Yokhin provides motivation for the combination from Yokhin's disclosure that x-ray fluorescence is a well-known technique for determining elemental compositions.

Applicant respectfully disagrees.

With regard to the Examiner providing motivation for combining Yokhin and Pirrung, Applicant submits that the motivation for combining Pirrung and Yokhin must come from the references, not from the Examiner. With regard to Yokhin providing the motivation, Applicant submits that Yokhin does not suggest or provide motivation for combining the teachings of Pirrung with those of Yokhin because Yokhin does not teach or suggest using x-ray fluorescence to detect chemical binding, and more particularly, Yokhin does not teach or suggest using x-ray fluorescence to detect chemical binding between binders and members of an array. The mere mentioning by Yokhin that x-ray fluorescence is a well-known technique for determining elemental compositions does not provide a suggestion or motivation to combine Yokhin with Pirrung. Yokhin does not suggest using x-ray fluorescence for any purpose other than what it had been already used for in the past, which does not include using x-ray fluorescence for detecting chemical binding, particularly for detecting binding between binders and members of a receptor array.

Applicant's argument that the invention does not require the constraint of labeling binders with additional optically fluorescent tags that could affect the binding properties of the chemical was not found persuasive according to the Office Action of December 30, 2005 because Pirrung discloses on page 35 lines 1-6 the option of using labeled or

unlabeled materials in the detection methods and therefore that Pirrung appears to offer the same benefits of the invention. Applicant respectfully disagrees. According to Pirrung on page 35 lines 1-6, for those cases where an unlabeled chemical is used, a labeled one is also used that binds to the unlabeled chemical. Labeled materials that bind to an unlabeled chemical that is already attached to a member of the array must also be used in order to detect binding using an analysis method disclosed in Pirrung. Thus, Pirrung still requires labeled materials.

Applicant further submits that even if there were a suggestion in the references to combine Yokhin and Pirrung, Yokhin does not teach how x-ray fluorescence would be combined with the teachings of Pirrung. Pirrung's method involves:

1) forming an array of receptors,
2) irradiating the array to activate it for binding, and thereafter,
3) exposing the array of activated receptors to binder(s), and
4) analyzing the array to see if chemical binding has occurred between any of the activated receptors and binder(s). According to Pirrung, this analysis is performed using autoradiographic (when the binder is radioactive), ultraviolet, or visible light techniques.

By contrast, Applicant's method involves:

1) forming an array of receptors,
2) exposing the array to binder(s),
3) irradiating the array to induce x-ray fluorescence signals;
4) analyzing the x-ray fluorescence signals to see if chemical binding has occurred between any of the receptors and binder(s).

In Applicant's method, step 1 (forming an array of receptors) and step 2 (exposing the array to binders) can be performed in any order, but step 3 (the irradiation step) must occur after step 1 and step 2. By contrast, Pirrung requires that irradiation happen after forming the array but before exposure to binders. Yokhin does not teach how x-ray fluorescence would be combined with Pirrung's method in a way that results in Applicant's claimed invention. For this reason, Applicant submits that the combination of Pirrung and Yokhin does not teach Applicant's claimed invention.

It should be noted that while Pirrung is silent with respect to the use of x-ray fluorescence to detect chemical binding, Pirrung is not completely silent with respect to x-rays. Pirrung teaches using x-rays to activate a receptor array by removing certain chemical groups, and it is the activated array that is used for subsequent binding studies. Pirrung does not use x-rays for x-ray fluorescence detection to detect chemical binding, and Yokhin's disclosure that x-ray fluorescence is used for elemental analysis does not provide a suggestion or motivation to use x-ray fluorescence to detect chemical binding between binders and arrayed receptors. For all of the above reasons, Applicant submits that claims 1-8, 10-18 and 20 are not obvious over Pirrung in view of Yokhin.

Applicant is submitting other evidence in further support of non-obviousness.

Applicant is providing the following two review articles: (1) Zhu et al., entitled "Protein Chip Technology," Current Opinion in Chemical Biology, vol. 7, issue 1, pp. 55-63, February 2003 (hereafter referred to as Zhu); and (2) Predki, entitled "Functional Protein Microarrays: Ripe for Discovery, Current Opinions in Chemical Biology, vol. 8, issue 1, February 2004 (hereafter referred to as Predki). These review articles are concerned with microarray technology for large-scale high-throughput biology. According to Zhu, microarray technology allows for fast, easy and parallel detection of many types of interactions such as, antibody-antigen, protein-protein, protein-nucleic acid, protein-lipid, and protein-small molecule, and enzyme-substrate interactions, and according to Zhu showed great potential for drug discovery. Zhu provides a listing on page 59, Table 2, of known (note that Zhu was published in February 2003) detection methods used in protein microarray experiments. These methods are ELISA, isotopic labeling, sandwich immunoassay, SPR, non-contact AFM, planar waveguide, SELDI, and electrochemical. Applicant respectfully notes that no method that uses x-ray fluorescence to detect chemical binding in a protein microarray is described on the list. Thus, Applicant submits that the presently claimed invention provides a new and unobvious method for the detection of chemical binding. Applicant submits that at the time Zhu was published, which was more than two years after the present patent application was filed, there was still a long-felt need for a highly sensitive label-free detection strategy for detecting chemical binding between binders and members of a

receptor array (in this case, a protein array), and that no one had yet employed, or even suggested, a strategy of using x-ray fluorescence to detect chemical binding between binders and members of a receptor array (in this case, an array of proteins).

Predki was published about one year after Zhu. According to Predki, page 8 at the bottom of column 1 through the top of column 2, protein arrays are expected to aid in developing "...meaningful insights and discovery in biology..." in areas that include "...molecular interactions for protein functional characterization to optimization of drug-protein interactions, from profiling enzyme substrates to profiling enzymatic activities. Functional protein microarrays clearly have the potential to make significant contributions to both basic and applied research...". On page 10, at column 2, under the heading of DETECTION, according to Predki, "...most applications of functional proteome microarrays for interaction or substrate detection have employed some type of labeling strategy; usually fluorescent...colorimetric...or radioactive...". Furthermore, "...although label-free technologies, such as surface plasmon resonance...mass spectrometry...and others, are highly desirable, their availability and sensitivity have not been high enough to have come into common use for functional protein microarrays...". Finally, Predki states that "...regardless of the physics employed, the development of practical, robust and sensitive, label-free detection strategies will be tremendously valuable...". Predki does not describe any method that uses x-ray fluorescence to detect chemical binding between binders and members of a microarray (a protein array, for example). Applicant submits that at the time Predki was published, which was more than three years after the present patent application was filed, there was still a long-felt need for a highly sensitive label-free detection strategy for detecting chemical binding between binders and members of a receptor array (in this case, a protein array), and that no one had yet employed, or even suggested, a strategy of using x-ray fluorescence to detect chemical binding between binders and members of a receptor array (in this case, an array of proteins).

As still further evidence of non-obviousness and of the long-felt need in the art for the presently claimed invention, Applicant is submitting a letter from Dr. Gregory Cuny, who is the Director of Medicinal Chemistry in the Laboratory for Drug Discovery in Neurodegeneration at Brigham & Women's Hospital, Harvard Medical School. The

letter was written in support of a submission for an R&D 100 award to MESA (Measurement of Enzyme-Substrate Affinities) technology, which is an embodiment of the presently claimed invention. According to Dr. Cuny, "...the measurement of protein-drug interactions is key to target-based drug development. These measurements are often difficult to obtain, especially when a mixture of proteins is used. "Label-free" fluorescence or fluorescence measurements obtained without the need for chemically appended fluorescing functional groups, is a significant improvement in the drug identification process. MESA technology (Measurement of Enzyme-Substrate Affinities) is an elegant solution to this unmet need for label-free drug measurement. It works by using x-ray excitation and x-ray fluorescence of heavy atoms. Many drugs contain these heavy atoms that are fluorescent in the x-ray spectrum. Label-free protein drug interaction measurements provide a means to answer questions concerning protein target identification during drug discovery and development. Utilizing phenotype cell-based assays is appealing from a drug discovery point of view. However, one drawback to this approach has been subsequent identification of the molecular target responsible for a particular compound's mechanism of action. Increasingly, regulatory agencies such as the FDA requires that the protein target be identified in order to grant Investigational New Drug (IND) states, i.e. approval for human clinical trials. Traditionally, this has involved chemical modification of the ligand with a fluorescent label. However, in many instances installation of the label results in diminished activity of the derivative compared to the parent molecule. MESA can allow label-free measurement of protein-drug interaction useful for target identification. This could unlock tremendous value by simplifying the process of target identification and would encourage increased utilization of cell-based assays in drug discovery. Label-free measurement of protein-drug interaction has been a longstanding need in the pharmaceutical industry. The preliminary MESA data is promising, and if it can be implemented on an industrial scale, it could significantly affect the development of new lifesaving drugs...". An R&D 100 award was received for the technology of the presently claimed invention in 2005. Applicant is also submitting a paper that describes the R&D 100 award to MESA.

As further support of non-obviousness, Benjamin P. Warner, the first named inventor acquired several million dollars in start-up capital, successfully competed for a license to the invention, and after a successful career at Los Alamos National Laboratory, separated from the laboratory and formed a company (CALDERA PHARMACEUTICALS, INC., hereafter referred to as CALDERA) that is using the invention. Currently, the company is located at the following address:

Caldera Pharmaceuticals, Inc.
3491 Trinity Drive, Suite B
Los Alamos, NM 87544
Phone: 505-412-2345
warner@caldera-pharmaceuticals.com.

Dr. Warner is the CEO and president of CALDERA. CALDERA has also received several million dollars of additional in funding for the construction of a major biotech facility in Los Alamos for the company. Such financing is relatively rare, and are given to promote economic development in New Mexico. CALDERA has grown to more than 10 employees in the past several months and this number is expected to increase to approximately 100 personnel. The commercial success of CALDERA is directly tied to the claimed invention, and this commercial success also shows that the invention addresses a long-felt need in the pharmaceutical industry.

For all of the above reasons, Applicant submits that claims 1-8, 10-18 and 20 are not obvious over Pirrung in view of Yokhin and Applicant urges that the rejection of claims 1-8, 10-18 and 20 under 35 U.S.C. §103(a) over Pirrung in view of Yohkin should be withdrawn.

According to the Office Action of June 3, 2004, claims 9 and 19 were finally rejected under 35 U.S. C. §103(a) over Pirrung et al. in view of Yokhin and further in view of Weinberg et al. (hereafter referred to as Weinberg).

Claim 9 is dependent from claim 1, and claim 19 is dependent from claim 11. For the above reasons, Applicant submits that claims 9 and 19 are not obvious over Pirrung in view of Yokhin and further in view of Weinberg. Applicant submits that Yokhin and Weinberg do not provide motivation or suggestion to modify Pirrung to arrive at a method of exposing an array of receptors binders, thereafter exposing the array to x-ray

radiation, thereafter detecting an x-ray fluorescence signal and using the detected x-ray fluorescence signal to determine whether or not binding has occurred. In view of the clear teachings of Applicant's application, the rejection of claim 9 under 35 U.S.C. §103(a) should be withdrawn.

Applicant respectfully requests that this amendment be entered into the present patent application.

For the reasons set forth above, Applicant believe that all currently pending claims are in condition for allowance, and such action at an early date is earnestly solicited. No new matter has been added by the above changes. Reexamination and reconsideration are respectfully requested.

Respectfully submitted,

Date: June 20, 2006

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CURRENT OPINION
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Protein chip technology

Heng Zhu* and Michael Snyder*†

Microarray technology has become a crucial tool for large-scale and high-throughput biology. It allows fast, easy and parallel detection of thousands of addressable elements in a single experiment. In the past few years, protein microarray technology has shown its great potential in basic research, diagnostics and drug discovery. It has been applied to analyse antibody–antigen, protein–protein, protein–nucleic-acid, protein–lipid and protein–small-molecule interactions, as well as enzyme–substrate interactions. Recent progress in the field of protein chips includes surface chemistry, capture molecule attachment, protein labeling and detection methods, high-throughput protein/antibody production, and applications to analyse entire proteomes.

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Abbreviations

AFM	atomic force microscope
GST	glutathione-S-transferase
PDMS	polydimethylsiloxane
PI	phosphatidylinositide
PVDF	poly(vinylidene fluoride)
SAM	self-assembled monolayer
SELDI	surface-enhanced laser desorption/ionization
SPR	surface plasmon resonance

Introduction

The past ten years have witnessed a fascinating growth in the field of large-scale and high-throughput biology, resulting in a new era of technology development and the collection and analysis of information. The challenges ahead are to elucidate the function of every encoded gene and protein in an organism and to understand the basic cellular events mediating complex processes and those causing diseases [1–4]. Miniaturized and parallel assay systems, especially microarray-based analyses, are crucial to large-scale and high-throughput biological analysis, as they are a rapid and economic way to interpret gene function [3,5,6], as demonstrated by DNA microarray approaches [7,8]. In a microarray format, capture molecules are immobilized in a very small area, and probed for various biochemical activities. High signal intensities and optimal signal-to-noise ratios can be achieved under ambient analyte conditions [3]. The microarray format has become the leading technology that enables fast, easy and parallel detection of thousands of addressable elements and side-by-side measurements.

Despite the success of DNA microarrays in gene expression profiling and mutation mapping, it is the activity of encoded proteins that directly manifest gene function. Thus, one would expect protein microarrays, in which proteins are prepared, arrayed and analysed at high spatial density, to be particularly powerful for analysing gene function, regulation and a variety of other applications. Proteins are more challenging to prepare for the microarray format than DNA, and protein functionality is often dependent on the state of proteins, such as post-translational modifications, partnership with other proteins, protein subcellular localization, and reversible covalent modifications (e.g. phosphorylation). Nonetheless, in recent years there have been considerable achievements in preparing microarrays containing over 100 proteins and even an entire proteome [1,2,9–11]. Alternative array formats have also been developed including tissue arrays [12], living cell arrays [13•,14•], peptide arrays [1,15–17,18••], antibody/antigen arrays [19••,20], protein arrays [21,22,23••–25••], carbohydrate arrays [26••,27••], and small-molecule arrays [28••]. However, technological challenges in the field of protein microarrays still remain.

In this review, we discuss recent progress in the field of protein chips, including surface chemistry, capture molecule attachment, protein labeling and detection methods, high-throughput protein/antibody production, and applications to analyse protein families and entire proteomes.

Manufacture of protein chips

It is important that protein chips retain proteins in an active state at high densities, are compatible with most commercial arrayers and scanners, and can be printed in such a fashion that the proteins remain in a moisturized environment. Soft substrates such as polystryrene, poly(vinylidene fluoride) (PVDF), and nitrocellulose membranes, which have been used to attach proteins in traditional biochemical analyses (e.g. immunoblot and phage display), are often not compatible for protein microarrays [2,16,22]. These surfaces often do not allow a suitable high protein density, the spotted material may spread on the surface, and/or they may not allow optimal signal to noise ratios [1,3,9,11]. Thus, most projects have

turned to using glass microscope slides or other materials that have been derivatized to attach proteins on their surface at high density. These slides have low fluorescence background and are compatible with most assays.

Different types of protein chips

A variety of types of chip have been designed, including 3D surface structures, nanowell and plain glass chips (Table 1). Polyacrylamide gel packet and agarose thin film microarrays, patterned by using photolithography technology on a glass surface, have been created by Guschin *et al.* [29] and Afanassiev *et al.* [30], respectively (Figure 1). Because both acrylamide and agarose form highly porous and hydrophilic matrixes, capture molecules, such as DNA, proteins and antibodies, can readily diffuse into the porous structure and are immobilized by cross-linking to the reactive ligands modified in the matrixes. Analytes are then added to these 3D arrays to carry out the biochemical assays [29]. Because of the formation of 3D matrixes on the glass surface, the capacity of protein immobilization is much higher than that on a 2D surface; the homogeneous water environment minimizes protein denaturing and thereby helps keep proteins in their active states. In addition to the sophisticated processes of creating such 3D matrixes, the major disadvantage of the 3D arrays is that it is more difficult to change buffers and recover trapped molecules from the matrix microarrays [4].

In contrast to 3D arrays, Zhu *et al.* [24••] fabricated an open structure, namely nanowells, on a polydimethylsiloxane (PDMS) surface supported by the standard glass slides. The nanowells significantly reduce evaporation and minimize cross-contamination and background. Because of the open nanowell structure, different components and buffers can be sequentially added, which is crucial for multiple-step biochemical assays. In addition, captured molecules can be easily recovered from the nanowells. When covered with gold in the nanowells, it is expected that high-throughput mass spectrometry and surface plasmon resonance (SPR) analyses can be performed. The biggest disadvantage of this technology it that specialized equipment is required to load the nanowells at high density.

Many groups now directly array proteins and antibodies/antigens onto plain glass surface [19••,20,23••,25••,31•,32]. To keep proteins in a wet environment during the printing process, high percent glycerol (30–40%) is used in sample buffer and the spotting is carried out in a humidity-controlled environment [23••,25••].

Surface chemistry

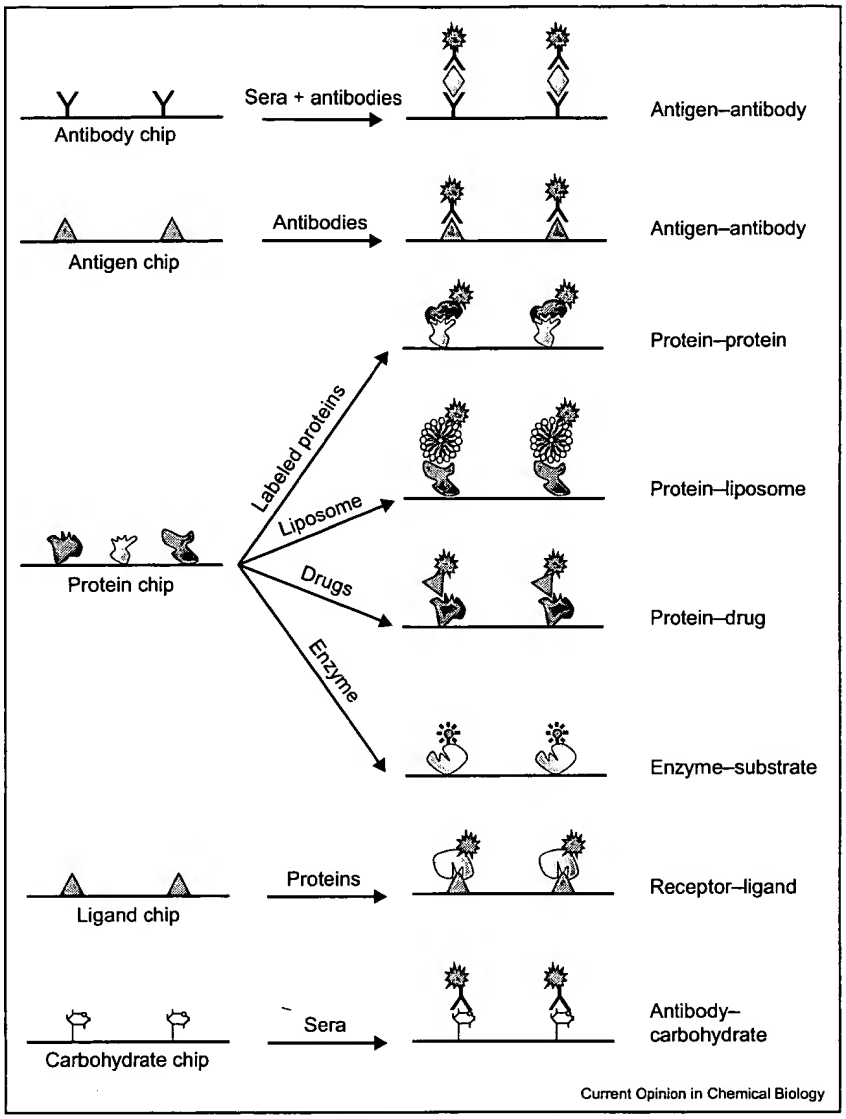
To attach proteins to a solid substrate, the surface of the substrate has to be modified to achieve the maximum binding capacity (Figure 2). A convenient method is to coat the glass surface with a thin nitrocellulose membrane or poly-L-lysine such that proteins can be passively adsorbed

Table 1

Comparison of current antibody/protein microarrays.

Surface	Attachment	Advantage	Disadvantage	References
PVDF	Adsorption and absorption	No protein modification requirement, high protein binding capacity	Non-specific protein attachment in random orientation	[2,16]
Nitrocellulose	Adsorption and absorption	No protein modification requirement, high protein binding capacity	Non-specific binding, high background Low-density arrays	[20,22]
Poly-lysine coated	Adsorption	No protein modification requirement	Non-specific adsorption	[19••]
Aldehyde-activated	Covalent cross-linking	High-density and strong protein attachment High-resolution detection methods available	Random orientation of surface-attached proteins	[23••,25••]
Epoxy-activated	Covalent cross-linking	High-density and strong protein attachment High-resolution detection methods available	Random orientation of surface-attached proteins	[24••]
Avidin coated	Affinity binding	Strong, specific and high-density protein attachment, low-background	Proteins have to be biotinylated	[58]
Ni-NTA coated	Affinity binding	Strong, specific and high-density protein attachment, low-background, uniform orientation of surface attached proteins	Proteins have to be Hisx6 tagged	[25••]
Gold-coated silicon	Covalent cross-linking	Strong and high-density protein attachment, low-background. Can be easily coupled with SPR and mass-spectrometry	Random orientation of surface attached proteins, tough to fabricate, not commercially available	[18••,35]
PDMS nanowell	Covalent cross-linking	Strong and high-density protein attachment, well suited for sophisticated biochemical analyses	Random orientation of surface attached proteins	[24••]
3D gel pad and agarose thin film	Diffusion	High protein binding capacity, no protein modification requirement	Tough to fabricate, not commercially available	[29,30]
DNA/RNA coated	Hybridization	Strong, specific and high-density protein attachment, low-background, uniform orientation of surface attached proteins	Sophisticated *in vitro* production of labeled proteins	[59]

Figure 1

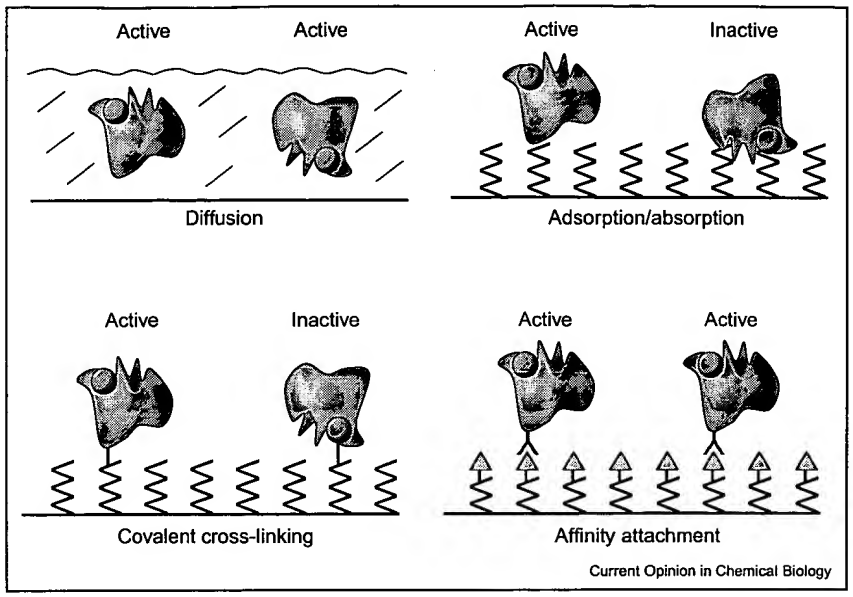


Applications of protein microarrays. There are two general types of protein microarray: analytical and functional protein microarrays. Analytical microarrays involve a high-density array of affinity reagents (e.g. antibodies or antigens) that are used for detecting proteins in a complex mixture. Functional protein chips are constructed by immobilizing large numbers of purified proteins on a solid surface. Unlike the antibody–antigen chips, protein chips have enormous potential in assaying for a wide range of biochemical activities (e.g. protein–protein, protein–lipid, protein–nucleic-acid, and enzyme–substrate interactions), as well as drug and drug target identification. Small molecule and carbohydrate microarrays are other types of analytical microarrays that have been demonstrated to be capable of studying protein binding activities to ligands and carbohydrates.

to the modified surface through non-specific interactions [20,22,33]. The attached proteins lay on the surface in random orientation and can be washed off under stringent washing conditions. In addition, the noise level is usually higher because of the non-specific adsorption/absorption.

To achieve more specific and stronger protein attachment, several groups have created reactive surfaces on glass that can covalently cross-link to proteins [23••–25••]. In general, a bifunctional silane cross-linker is used to form a self-assembled monolayer (SAM), which has one functional group that reacts with the hydroxyl groups on glass surface, and another free one that can either directly react with primary amine groups of proteins (i.e. aldehyde or epoxy groups) or can be further chemically modified to reach maximum specificity [28••,34]. Gold-coated glass surface is another variation [18••,35]. To form a SAM on gold surface, bifunctional thio-alkylene is usually used,

Figure 2



Comparison of different protein attachment methods. Proteins can be attached to various kinds of surface via diffusion, adsorption/absorption, covalent cross-linking and affinity interaction. Except affinity attachment, proteins are usually laid on the surface in a random fashion, which may alter the native conformation of proteins, reduce the activity of proteins, or make them inaccessible to probes. However, when proteins are attached to the surface via their affinity tags, it is very likely that every protein molecule uniformly attaches to the surface and, therefore, proteins are more likely to remain in their native conformation, while the analytes have easier access to the active sites (indicated by the red dots) of proteins.

which has a SH-group that reacts with gold, and another free one that reacts with capture molecules. The advantage of using gold-coated surface is that SPR and mass spectrometry can potentially be integrated as detection methods to monitor the dynamics of the reactions, or to identify the captured molecules, respectively [18••,35,36]. This approach provides the opportunity to study dynamics of biochemical reactions in a high-throughput fashion, and has great potential in drug and drug-target discovery and biomedical research [36].

In the above covalent cross-linking approaches, because the reactive ligands also exist in the side chains of proteins, it is plausible that the attached proteins attach to the surface in a random fashion, which may alter the native conformation of proteins, reduce the activity of proteins, or make them inaccessible to probes (Figure 2). Perhaps the best means of protein attachment is through highly specific affinity interactions [3,24••,25••]. Proteins fused with a high-affinity tag at their amino or carboxy terminus are linked to the surface of the chip via this tag, and hence, all of the attached proteins should orient uniformly away from the surface (Figure 2) [25••]. Using this method, immobilized proteins/antibodies are more likely to remain in their native conformation, while the analytes have easier access to the active sites of proteins. This approach was first successfully demonstrated in attaching 5800 fusion proteins containing a His tag onto a nickel-coated glass slide [25••]. It should also be possible to use other affinity methods such as glutathione/glutathione-*S*-transferase (GST) and phosphonate/serine esterase cutinase ligand/protein tags [37].

Protein delivery systems

Although a 96-format dot blot instrument has been used to create low-density protein arrays on filters [9,22,33], high-density protein microarrays (>30 000 spots per slide) can be achieved using robotic contact printing tools, such as those developed for creating DNA microarrays [23••,25••]. The contact printing arrayers deliver sub-nanoliter sample volume directly to the surface using tiny pins with or without capillary slots. Because these contact printing robots cannot align their pins to the pre-fabricated structures and need to touch the surface, non-contact robotic printers, which use ink-jet technology, were used to deposit nanoliter to picoliter protein droplets to polyacrylamide gel packets [21] and nanowells [24••]. Although the current Packard ink-jet microarrayer can be slowed when spotting many different samples and the shearing force during drop formation may damage some samples [1], it is not restricted to the surface structure and is well suited for more complicated biochemical assays. Recently, electrospray deposition technology was applied to deliver dry proteins to a dextran-grafted surface [38]. This technology further reduced the spot size from ~150 μm to ~30 μm.

Table 2

Summary of current detection methods used in protein microarray experiments.

Detection	Probe labeling	Data acquirement	Real time	Resolution	References
ELISA	Enzyme-linked antibodies	CCD imaging	No	Low	[20,28••,38]
Isotropic labeling	Radio isotope-labeled analyte	X-ray film or phosphoimager	No	High	[22,23••,24••]
Sandwich immunoassay	Fluorescently labeled antibodies	Laser scanning	No	High	[20]
SPR	Not necessary	Refractive index change	Yes	Low	[41–44]
Non-contact AFM	Not necessary	Surface topological change	No	High	[40]
Planar waveguide	Fluorescently labeled antibodies	CCD imaging	Yes	High	[45]
SELDI	Not necessary	Mass spectrometry	No	Low	[39]
Electro-chemical	Metal-coupled analyte	Conductivity measurement	Yes	Medium	[60]

Probe detection methods

Fluorescence detection methods are generally the preferred detection method (Table 2) because they are simple, safe, extremely sensitive and can have very high resolution [1,3]. They are also compatible with standard microarray scanners. Typically, a chip is either directly probed with a fluorescent molecule (e.g. protein or small molecule) or in two step by first using a tagged probe (e.g. biotin), which can then be detected in a second step using a fluorescently labeled affinity reagent (e.g. streptavidin). Another fluorescent labeling method is rolling circle amplification (RCA), which is extremely sensitive [39]. However, other detection methods can also be used. For example, ELISA was first used to detect proteins for both filter arrays [20,40] and glass arrays [29]. Ge [22], and Zhu *et al.* [24••] have used radioisotope labeling to study protein–protein, protein–DNA, protein–drug interactions on filter arrays, and kinase–substrate interactions in nanowells, respectively.

Because labeling molecules can sometimes affect protein activity and are restricted to the available detection channels, non-labeling methods have advantages as a direct detection approach for antibody microarrays. SELDI (surface-enhanced laser desorption/ionization) mass spectrometry has been used to detect low-density arrays of captured proteins [41]. Captured proteins on an array of metal surface (SELDI protein array) were vapourized using a laser beam, followed by the analysis of mass spectrometry data to reveal the identities of these proteins. The atomic force microscopy (AFM) method takes advantage of surface topological changes to identify the captured proteins on an antibody array [42]. When the immobilized rabbit IgG on a gold surface bound to its complimentary antibodies, goat ant-rabbit IgG, AFM could detect the height increase, and therefore, revealed the binding activities.

To study the kinetics of antigen–antibody interactions, however, real-time detection methods will be useful. SPR has matured as a versatile detection tool to study the kinetics of receptor–ligand interactions with a wide range of molecular weights, affinities and binding rates [43–45]. Although the commercially available SPR chips are limited to a few channels, Myszka and Rich [46] described a sensor surface with 64 individual immobilization sites in a single flow cell. Alternatively, Sapsford *et al.* [47] developed an antibody array biosenor to study the kinetics of antigen binding using a planar waveguide as the detection method. More importantly, they demonstrated that significant signal intensity could be achieved from spots as small as 200 μm in diameter. It is therefore expected that the latter approach is well suited for high-throughput and parallel kinetics studies.

Two functional classes of protein microarrays

There are two general types of protein microarrays. Firstly, analytical microarrays in which antibodies, antibody mimics or other proteins are arrayed and used to measure the presence and concentrations of proteins in a complex mixtures. Secondly, functional protein microarrays, in which sets of proteins or even an entire proteome are prepared and arrayed for a wide range of biochemical activities.

Analytical microarrays

Analytical microarrays involve a high density array of affinity reagents that are used for detecting proteins in a complex mixture. They have enormous potential for monitoring protein expression on a large-scale, a process that is sometimes termed protein profiling.

Antibody microarrays

The most common form of analytical arrays are antibodies/antibody mimic arrays in which antibodies (or similar reagents) that bind specific antigens are arrayed on a glass slide at high density. A lysate is passed over the array and the bound antigen is detected after washing. Detection is usually carried out by using labeled lysates or using a second antibody that recognizes the antigen of interest. The biggest challenge with these methods is producing reagents that identify the protein of interest and with high enough specificity in a high-throughput fashion.

Antibodies are the traditional reagent of choice for detecting proteins in complex mixtures. However, polyclonal sera are often not specific and are expensive to produce,

and the conventional hybridoma method of producing highly specific monoclonal antibodies is also time-consuming, laborious and costly. Recently, alternative methods, such as phage antibody-display, ribosome display, SELEX (systematic evolution of ligands by exponential enrichment), mRNA display, and affibody display, have been developed to expedite the production of antibodies and/or antibody mimics [1–3,9]. All of these approaches involve the construction of large repertoires of viable regions with potential binding activity, which are then selected by multiple rounds of affinity purifications. The binding affinity of the resulting candidate clones can be further improved using maturation strategies. However, the ideal selection system, which is not only fast, robust, sensitive, and of low cost, but automated and minimized, is yet to be fully developed [3,9].

In spite of the challenge in obtaining specific antibodies, several studies using antibodies have recently appeared. In one of the largest studies to date, Sreekumar *et al.* [31•] spotted 146 distinct antibodies on glass to monitor the alternations of protein quantity in LoVo colon carcinoma cells. Their results revealed radiation-induced up-regulation of many interesting proteins, including p53, DNA fragmentation factor 40 and 45, tumour necrosis factor-related ligand, as well as down-regulated proteins.

The most significant problem with antibody arrays is specificity. Proteins are often present in a very large dynamic range (10^6); thus, reagents that might have high affinity for one protein, but are low affinity for another will still exhibit detection of the lower affinity protein if it is much more prevalent. Haab *et al.* [19••] have investigated the ability of 115 well-characterized antibody–antigen pairs to react in high-density microarrays on modified glass slides. 30% of the pairs showed the expected linear relationships, indicating that a fraction of the antibodies were suitable for quantitative analysis. To avoid this problem, many groups have turned to using sandwich assays, in which the first antibody is spotted on the array and then the antigen is detected with a second antibody that recognizes a different part of the proteins. This approach dramatically increases the specificity of the antigen detection, but required that a least two high-quality antibodies exist for each antigen to be detected.

Other analytical microarrays

In addition to antibody microarrays, other analytical microarrays have been developed. These include microarrays for profiling antibodies in a patient's serum, essentially the reciprocal of that described above. Joos and colleagues [20] used 18 diagnostic markers for autoimmune diseases to form an autogen microarray and screened for antigen–antibody interactions. Hiller *et al.* [48] arrayed 94 purified allergen molecules, which included most common allergen sources, on glass slides to miniaturize the allergy test. These allergen molecules were not restricted to proteins, but also included peptides and small molecules. The allergen microarrays were specifically used to determine and monitor allergic patients' IgE reactivity profiles to large numbers of disease-causing allergens in single measurements. Only minute amounts of serum were required. Potential new leads to allergic diseases were revealed, and some of them have been confirmed using the traditional skin tests. To characterize autoantibody responses, Robinson *et al.* [49] robotically arrayed hundreds of autoantigens, including proteins, peptides, and other biomolecules, in eight distinct human autoimmune diseases onto glass slides to form the autoantigen microarrays. These arrays were incubated with patient serum samples to define the pathogenesis of autoantibody responses in human autoimmune diseases. To explore the possibility of quantitative measurement of serum-specific IgE using protein chip format, Kim *et al.* [50] used purified dermatophagoides pteronyssinus (Dp)-specific IgE to detect allergens in serum challenged with Dp, egg white, milk, soybean and wheat. These authors were able to demonstrate that quantitative measurement of allergen in a protein mixture could be achieved.

Functional protein chips

Functional protein chips are constructed by immobilizing large numbers of purified proteins on a solid surface. Unlike the antibody chips, which are mainly developed for diagnostics and profiling of protein and epitope expression, protein chips have enormous potential in basic research, as well as drug and drug target identification (Figure 1). For example, both the Mrksich [18••] and Schreiber [23••] groups have demonstrated the potential of using protein microarrays to conduct enzymatic assays to identify downstream targets of kinases. However, the first great obstacle to overcome is the purification of large numbers of proteins in a high-throughput manner.

High-throughput protein production

To analyse the biochemical activities of as many proteins as possible, many research groups and companies have contributed tremendous effort in developing high-throughput protein purification methods. The combination of recombinant proteins and affinity purification has been used to purify proteins from various host cells, including lines from *Escherichia coli*, yeast, insects and humans [9,25••,40,51,52].

Leuking *et al.* [11] cloned cDNAs from human fetal brain tissues as C-terminal Hisx6-tagged fusions. The Hisx6 tags were used first as an indicator of in-frame fusion proteins and then served as an affinity tag for high-throughput protein purification from *E. coli*. In a later report, LaBaer and colleagues [51] created a system (FLEXP) that performs from cDNA cloning to protein production from *E. coli* in a fully automated fashion. In a test case, ~80% of 336 random cDNA clones could

successfully purify fusion proteins in full length. Because the purification process was automated, at least 1000 proteins could be purified in one day. However, because eukaryotic proteins expressed in prokaryotic systems are not post-translationally modified, our group has developed a high-throughout protein purification method from the budding yeast [25••]. The yeast genes were cloned as N-terminal GST:Hisx6 fusions, and purified using the GST affinity tags. In two weeks, >6500 yeast proteins could be purified individually from 3 ml culture. For the same reason, Albala *et al.* [52] chose 72 unique human cDNA clones to create an array of recombinant baculoviruses, from which 42% of the clones produced soluble fusion proteins in a 96-well format.

Alternatively, proteins can be produced using cell-free expression systems. For example, Keefe and Szostak [53] established a mRNA display system, in which each protein was *in vitro* translated and covalently linked through its carboxy terminus to the 3′ end of its coding mRNA. More interestingly, He and Taussig [54] created a protein *in situ* array (PISA), which combines the protein production and immobilization in one step. Although the experiment was performed in microtiter dishes, it is plausible that the system can be easily automated and applied to a microarray format.

Applications of functional protein chips

Functional protein chips like traditional assays performed in microtiter plates [55] are suitable for a wide variety of biochemical analyses. Unlike microtiter plates, however, they are much more amenable to high-throughput studies and use small amounts of reagents. In early proof-of-concept studies, MacBeath and Schreiber [23••] fabricated protein microarrays with three purified proteins at high density, and performed protein–protein, protein–ligand, and kinase–substrate interactions using three test systems. Likewise, Mirzabekov and co-workers [29] demonstrated that proteins immobilized in the gel pads could still show their enzymatic activities.

Studies analysing large sets of proteins have recently been performed. Using a PDMS nanowell chip mounted on glass slides, Zhu *et al.* [24••] analysed the activity of 119 yeast kinases for 17 different substrates. The substrates were first covalently immobilized to individual nanowells, and individual protein kinases with radio-labeled ATP were incubated with the substrates. After washing away the kinases and unincorporated ATP, the nanowell chips were analysed for phosphorylated substrates using a phosphoimager. Not only known kinase–substrate interactions were identified, but also many novel activities were revealed. This included the unexpected discovery that one-fourth of yeast protein kinases are capable of phosphorylating their substrates on tyrosine, even though the kinases are members of the Ser–Thr family of protein kinases.

Because the ultimate goal of proteomics is to study biochemical activities of every protein encoded by an organism, Zhu *et al.* [25••] prepared the first proteome chip. They cloned ~94% (>5800 of 6200) of the yeast open reading frames in a yeast expression vector that expresses the proteins as N-terminal GST-Hisx6 double tagged fusions and developed a high-throughput yeast protein purification method to individually purify proteins. 80% of yeast proteins are full length and of sufficient quantities that they are detectable by most assays. The proteins were purified using the GST tags and were then attached to Ni-NTA-coated glass slides using the HisX6 tags. In our initial study, the chips were probed with Cy3-labeled calmodulin and various phosphatidylinositides (PIs). Calmodulin is a highly conserved calcium-binding protein that regulates many signaling pathways and has many known binding partners. In addition to identifying known interactions, 33 novel binding proteins were detected. Sequence comparison revealed a novel binding motif that was related, but distinct from, the previous known calmodulin-binding motif. To demonstrate that proteome chips could be used to globally probe for novel activities, the chips were incubated with five different PIs, which are important secondary messengers that regulate diverse cellular processes [56]. 150 novel lipid-binding proteins were identified, 49 of which exhibited preferential binding to PIs. These results convincingly showed that proteins immobilized on a surface were able to bind to low molecular weight compounds. This suggests that an entire proteome can be immobilized on a glass surface to directly screen for interactions with proteins and small molecules.

Peptide arrays

It is of great interest and importance to identify epitopes in proteins that define the core activity. To study the substrates of the nonreceptor tyrosine kinase *c-Src*, Houseman *et al.* [18••] immobilized 9-mer peptide substrates on a gold-coated glass surface to form a high-density peptide microarray, and characterized the phosphorylation of the peptide using SPR, fluorescence and phosphoimaging. They could also quantitatively evaluate the effect of three known inhibitors of the kinase. Although their work was still primitive, the authors demonstrated the potentials of coupling peptide chips with various detection methods to quantitatively study dynamics of enzyme–substrate interactions, and applications in drug discovery. Our group has also designed 20 17-mer peptide substrates and covalently immobilized them to epoxy-activated glass surface (unpublished data). 120 yeast kinases were screened for their preferred substrates.

Because peptides are much shorter and more stable than proteins, high-density peptide microarrays can be fabricated by direct synthesis of peptides on a surface using photolithography or light-directed synthesis [15,57].

These approaches greatly save cost in peptide synthesis because only a tiny amount of material is needed.

Conclusion

Protein microarrays are poised to become one of the most powerful tools in the field of large-scale biology because of their enormous potential in basic research, diagnostics and drug discovery. High-density robotically spotted protein microarrays on glass have been validated to analyse an entire proteome and hold great promise for high-throughput discovery applications [19••,23••,25••]. Improvements in generating large sets of antibody reagents, recombinant proteins from a variety of host cells and other types of capture molecule will further increase the interest in this field.

The fast growth of protein microarray technologies is fueled by the continuous growth of genomic information. The actual impact of these new technologies for proteomic and medical research, drug discovery and clinical diagnostics are yet to be fully realized. It is expected that the integration of large datasets from different approaches will result in the generation of a huge network and deepen our understanding of the molecular mechanisms of life.

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Functional protein microarrays: ripe for discovery

Paul F Predki

The manufacture and use of protein microarrays with correctly folded and functional content presents significant challenges. Despite this, the feasibility and utility of such undertakings are now clear, and exciting progress has recently been demonstrated in the areas of content generation, printing strategies and protein immobilization. More importantly, we are now beginning to enjoy the fruits of these efforts as functional protein microarrays are being increasingly employed for biological discovery purposes. Recent examples of this include the characterization of autoantibody responses, antibody specificity profiling, protein–protein domain interaction profiling and a comprehensive characterization of coiled-coil interactions. The best, however, is yet to come.

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Introduction

The human genome project has catalyzed the development of new large-scale approaches to addressing biological questions. A prime example of this is the now common use of DNA microarrays for large-scale mRNA expression analysis. Functional protein arrays (microarrays with immobilized functional proteins, Figure 1) are a logical extension of DNA microarrays. However, the manufacture and use these microarrays presents significant challenges compared with their DNA counterparts. In fact, before the first report [1••], many doubted it could even be done at all!

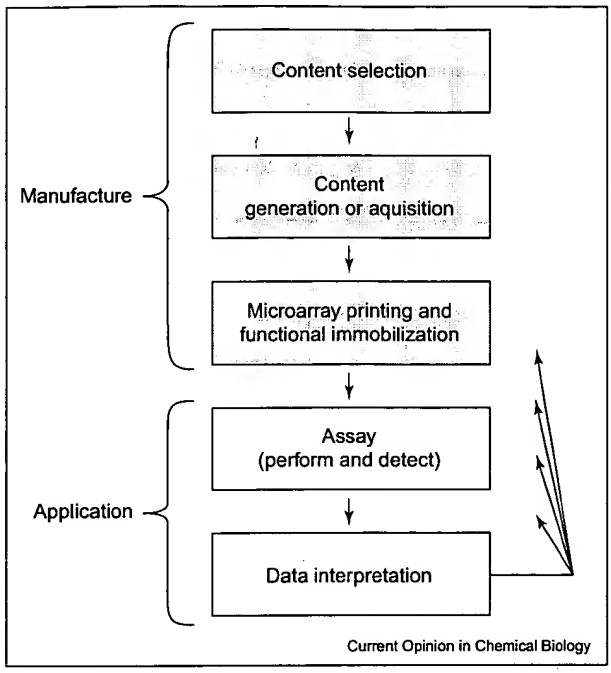
While tractable, the manufacture of protein microarrays is not for the faint of heart. Considerable challenges still exist in terms of content generation, printing, functional immobilizing, and detection. The current state and recent advances in each of these areas is briefly reviewed here. The end goal of all of this research, however, is its application towards developing meaningful insights and discovery in biology. These applications span a wide range, from molecular interactions for protein functional characterization to optimization of drug–protein interactions, from profiling of enzyme substrates to profiling enzymatic activities (Table 1). Functional protein microarrays clearly have the potential to make significant contributions to both basic and applied research [2]. Although many of these applications remain at the proof-of-concept stage, there have recently been important advances in reducing concept to practice, which I review here.

Functional content

Perhaps the most significant barrier to manufacture of functional protein microarrays is generation of the protein content itself. Early efforts in creating protein expression collections focused on more random approaches, such as cDNA libraries cloned into *Escherichia coli* expression vectors [3]. In fact, the use of such collections in protein microarrays has recently been described [4]. However, a significant drawback of such approaches is the relatively low yield of folded full-length proteins, even in the presence of positive selection. More focused efforts to express a random set of human proteins in *E. coli* have given reasonable yields of ~60%; ~80% of which could be purified under non-denaturing conditions [5]. Unfortunately, proteins expressed in *E. coli* lack the post-translational modifications observed in eukaryotic proteins, which can be required for proper protein function. Insect cells provide an example of a eukaryotic expression system successfully adapted to high-throughput protein expression [6]. In recent experiments, approximately 15% of human proteins expressed in insect cells showed detectable levels of phosphorylation (Figure 2). High-throughput expression of human proteins in COS cells (a cell line established from monkey kidney cells) has also been reported [7]. In practice, different proteins often require different expression hosts and vector constructs for optimal expression. Given this need, an ability to readily shuttle inserts from one vector to another will be important. Commercial systems such as Invitrogen's Gateway and BD Clontech's Creator systems meet this requirement, although licensing restrictions with commercial systems can be problematic. Expression clone sets such as the human FLEXgene repository [8,9], which is primarily generated from the Mammalian Gene Collection [10], and the *Caenorhabditis elegans* ORFeome collection [11••] were generated in such systems.

An alternative to expression clones, cell-free expression, is not reviewed here, but has apparently been adapted to 96-well format with good success [12], and therefore shows promise for the highly parallel protein expression capabilities required to generate protein microarrays. Regardless of the mode of expression, however, there is a subsequent need to purify large numbers of different proteins in parallel with reasonable yields. While a

Figure 1



Manufacture and application of functional protein microarrays. This high-level flowchart summarizes the major steps required in the manufacture and use of functional protein microarrays.

96-well plate format makes this easy to do in principal [5–7], doing it well is a significant, albeit surmountable, engineering challenge.

Microarray printing

There are two general approaches to microarray printing: contact and non-contact. Given the requirement to array large numbers of different proteins, contact printing is currently the most suitable choice, although non-contact printing of functional protein microarrays is certainly possible [13].

Recent advances in microarray printing include a laser transfer technique [14], microfabricated fountain pens for high-density array construction [15], as well as a novel affinity contact printing procedure employing a multi-use stamp [16]. Cooks' group at Purdue University recently described an exciting proof-of-concept using electrospray ionization of a protein mixture followed by mass ion separation and sequential soft landing deposition onto a surface to create a protein array [17••]. This technology, while promising, has many challenges ahead, including improving print speed as well as addressing protein quantity, identity and functionality.

Functional immobilization

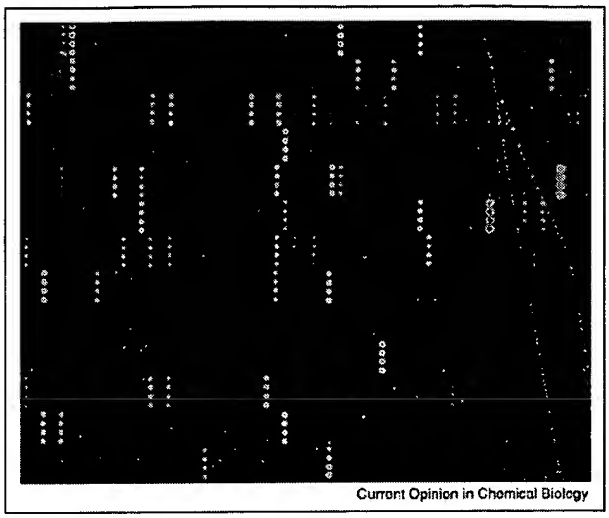
Basic strategies for protein immobilization consider covalent versus noncovalent and oriented versus random

Table 1

Applications of functional protein microarrays. A summary of many of the basic and drug research applications of functional microarray experiments.

Experiment	Basic research application	Drug research application
Protein–protein interaction profiling	Pathway mapping Protein interaction mapping Protein function determination K_D estimation	Target discovery Early target validation
Protein–lipid interaction profiling	Pathway mapping Protein function determination	Target discovery Early target validation
Protein–DNA interaction profiling	Pathway mapping DNA-binding protein discovery	Target discovery Early target validation
Protein–small-molecule interaction profiling	Pathway mapping Metabolomics	Drug rescue Target/mechanism determination Alternate target identification Specificity profiling IC_{50} determination Lead optimization
Substrate assays	Pathway mapping Substrate identification	Target discovery Early target validation
Enzymatic activity profiling	Enzyme activity discovery	Target discovery Early target validation
Enzymatic activity assay	Enzyme kinetics	Specificity profiling IC_{50} determination Lead optimization

Figure 2



Phosphorylation of human proteins expressed in insect cells. Approximately 1000 different human proteins expressed in insect cells were spotted onto a microarray in quadruplicate. Phosphorylation was detected by the phosphate binding dye Pro-Q™ (Molecular Probes). Approximately 15% of proteins showed detectable signal. A portion of the image is shown.

attachment, as well as the nature of the surface itself. Some examples of oriented attachment include immobilization of His-tagged proteins on nickel slides [1••] and of biotinylated proteins to avidin-coated slides [18•]. There is evidence that, for some proteins, oriented attachment enhances the fraction of available and/or active protein [19]. However, several demonstrations of functional proteins using random attachment have now been published [20,21], including most of those in the 'New applications' section [1••,4,22,23,24•,25••,26•,27••,28]. Similarly, successful use of microarrays generated by both covalent and noncovalent attachment has been reported. Also, a large number of surfaces have been demonstrated to be compatible with functional protein microarrays, from acrylamide- and nitrocellulose-coated to aldehyde and poly-L-lysine modified glass slides. Self-assembled monolayers provide yet another technique for the development of biocompatible surfaces [29,30]. G-protein-coupled receptors have even been functionally microarrayed onto γ-aminopropylsilane slides [31–33]. Recent developments in lipid immobilization may provide improved capabilities in membrane protein immobilization [34–36].

An ideal surface or immobilization for all proteins and all applications doesn't exist, so work to develop biocompatible surfaces is both ongoing [33] and important. However, as is demonstrated in the literature, it is clear that a large number of proteins retain functionality over a wide range of surface and immobilization conditions. Thus, despite the lack of an ideal universal surface or immobilization approach, existing methods are more than adequate for many applications.

Detection

Most applications of functional proteome microarrays for interaction or substrate detection have employed some type of labeling strategy; usually fluorescent [1••,4,23,24•, 25••,26•,28,37,38], colorimetric [22] or radioactive [23,39]. One noteworthy development in fluorescent protein labeling is the puromycin-based labeling strategy [40,41], which enables fluorescent labeling simultaneously with cell-free expression. Although label-free detection technologies, such as surface plasmon resonance [42], mass spectrometry [43] and others, are highly desirable, their availability and sensitivity have not been high enough to have come into common use for functional protein microarrays. An interesting development in label-free detection is an alamethicin-based detection strategy that measures the blocking of channel current through a planar lipid bilayer upon binding [44]. Regardless of the physics employed, the development of practical, robust and sensitive label-free detection strategies will be tremendously valuable.

Recent applications

The early literature in the functional protein microarray field consists primarily of proof-of-concept work. The pioneering paper of MacBeath and Schreiber, for instance, demonstrated three important proofs for protein microarrays; protein–protein binding, protein kinase substrate phosphorylation and small-molecule–protein binding [23]. Around the same time, modified polyacrylamide gel pads were demonstrated for protein immobilization and subsequent immunoassay and enzymatic kinetic measurements (horseradish peroxidase, alkaline phosphatase, β-D-glucuronidase in the presence or absence of inhibitors) [22]. More recent work from Yao's laboratory has demonstrated the novel application of mechanism-based inhibitors for activity-based detection of enzymes using protein microarrays [37].

The first reported use of functional protein microarrays for biological discovery purposes was reported by Snyder's laboratory, which manufactured and used yeast proteome microarrays for protein interaction and lipid binding screens [1••]. Since that time, a slowly increasing number of discovery-based papers have been published.

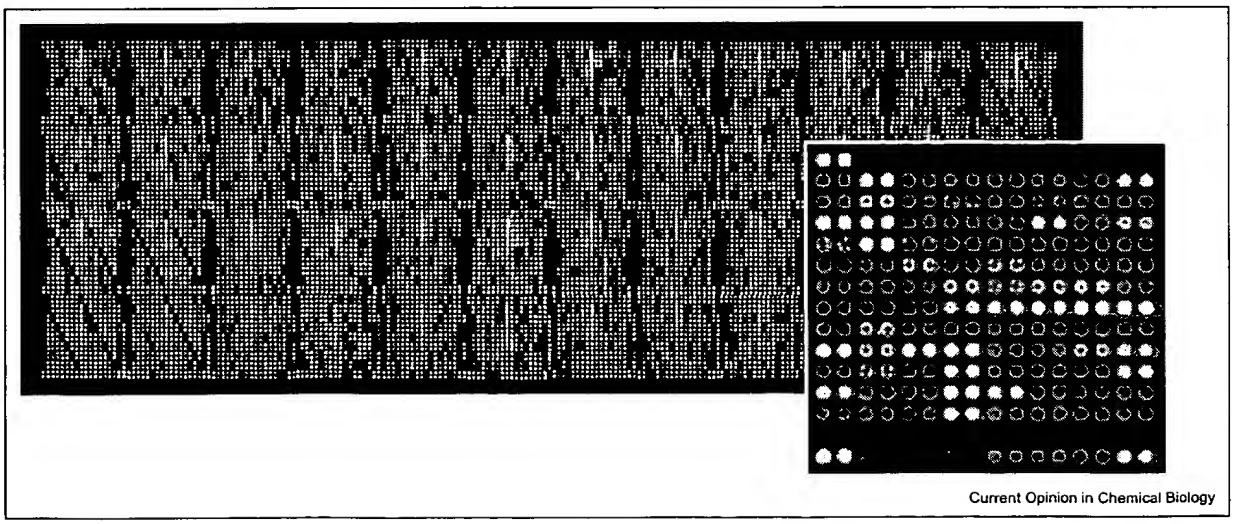
In 2002, Espejo *et al.* [24•] reported the use of protein domain microarrays to identify novel protein–protein interactions. In this study, peptide motifs were used to demonstrate proper binding specificity of several glutathione S-transferase fusions of protein interaction modules, such as WW, SH (Src homology), forkhead-associated, plekstrin homology and FF domains. The arrays were generated by microarraying proteins at 1 mg/ml onto

nitrocellulose-coated glass slides, and were probed with biotinylated peptides prebound to fluorescently labeled streptavidin. The same type of slides were also probed with total-cell lysates, re-probed with an antibody raised against either Sam68 or SmB′, and detected with fluorescently labeled secondary antibody. Whereas Sam68 displayed the expected binding partners, SmB′ showed an unexpected degree of binding to SH3 domains, leading the authors to hypothesize a role for SmB′ as a spliceosome attachment scaffold.

More recently, Newman and Keating [25••] used protein arrays to test all binary interactions of 49 (out of 55 known) coiled coil strands from human basic-region leucine zipper transcription factors. After high-performance liquid chromatography purification, the peptides were arrayed in quadruplicate onto aldehyde-derivatized glass slides. To reduce homodimerization, reduced and guanidine hydrochloride denatured peptides were printed and probed with denatured fluorescent peptides, which were rapidly diluted immediately before use. Both strong (~50 nM) and weak (~3 μM) binding could be detected, and high specificity was observed in the experiments, with only ~14% of all measured pairs showing interactions, and only ~6% showing strong interactions. The array results agreed well with follow-up circular dichroism studies. As expected, peptides within families tended to show similar interactions, whereas peptides from different families had distinct interactions. In addition, several previously undetected interactions were reported, permitting the generation of some exciting biological hypotheses. It will be interesting to watch these and other hypotheses be tested in the near future.

A proof-of-concept experiment for antibody specificity profiling was recently published by Lueking *et al.* [4]. Polyacrylamide-coated glass slides were used to create microarrays of ~2400 human fetal brain cDNA expression clones that express protein in *E. coli*. These microarrays were used to probe with mouse monoclonal α-GAPDH (glyceraldehydes phosphate dehydrogenase) and α-HSP90β (heat-shock protein) antibodies. Both antibodies preferentially recognized their cognate antigens, but additional unrelated cross-reactive proteins were also identified. In a similar study employing 96 *Arabidopsis* proteins, only specific antigen binding was observed [38]. It is likely, however, that most of the proteins used in these studies were unfolded. An example of antibody specificity profiling with whole-proteome microarrays using folded proteins was reported by Michaud *et al.* [45•]. In this case, both polyclonal and monoclonal antibodies generated towards yeast proteins were probed against a yeast proteome microarray. Not surprisingly, monoclonal antibodies tended to show more specificity than polyclonal antibodies. However, even monoclonal antibodies exhibited demonstrable cross-reactivity. Interestingly, most cross-reactivity could not be predicted *a priori* on the basis of sequence analysis, suggesting that empirical approaches to profiling antibody specificity should be an important consideration when developing or using antibodies for research or medical purposes. This new approach to profiling antibody specificity will

Figure 3



Fluorescent Image of a Yeast ProtoArray™. Microscope slides were spotted with >4000 different proteins cloned from and expressed in yeast. Proteins were detected using a Cy5-labeled antibody directed against an epitope tag. Slides were scanned using an Axon 4000B microarray laser scanner. The scan of an entire 48-subarray slide is shown at the top of the figure, while an image of just one subarray is shown in the bottom. Copyright 2003 Protometrix Inc. Reprinted with permission.

become increasingly powerful as larger sets of proteins become available.

Robinson *et al.* [26•] reported the use of autoantigen arrays for screening human disease sera. Microarrays were constructed using 196 autoantigens (including proteins, protein complexes, peptides and DNA) corresponding to eight different autoimmune diseases. These autoantigens were spotted onto 1152 features on poly-L-lysine-coated slides and probed with fluorescently labeled human serum. Using this approach, the authors observed distinct autoantibody profiles consistent with each disease. In addition, they demonstrated the ability to use these microarrays for epitope mapping of autoantibody response. In follow-up work, 'myelin proteome' microarrays were used to characterize autoantibody epitope spreading in experimental autoimmune encephalomyelitis, a mouse model for multiple sclerosis [28]. Microarray analysis was subsequently used to guide the development of tolerizing vaccines. This work has clearly demonstrated the potential of autoantigen arrays in characterizing and ultimately treating autoimmune disease. The approach will ultimately be supplemented by the identification of currently unknown autoimmune antigens. As an early example of this, Lueking *et al.* recently reported screening low complexity human protein arrays against autoimmune sera [4], identifying some interesting leads for further analysis. Finally, a 430-peptide/protein simian-human immunodeficiency virus microarray was recently used to profile macaque immune response to vaccination, with results predictive of survival [27••]. In addition, three novel viral epitopes were identified.

Conclusions

Functional protein microarrays hold enormous potential for biological discovery and drug development. Significant attention has been and will continue to be devoted to technology development. However, several recent literature publications highlight the fact that, despite continuing technological challenges, the current state of the art is such that this huge potential is now being unleashed. The imminent commercial introduction of functional protein microarrays [46] (Figure 3) will only accelerate this process.

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- •• of outstanding interest

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February 14, 2005

R&D100 Judges
c/o Los Alamos National Laboratory
Los Alamos, NM 87545

Dear R&D100 Judges:

I am writing to support the "MESA" technology R&D100 entry. The measurement of protein-drug interactions is key to target-based drug development. These measurements often are difficult to obtain, especially when a mixture of proteins is used. "Label-free" fluorescence or fluorescence measurements obtained without the need for chemically appended fluorescing functional groups, is a significant improvement in the drug identification process.

MESA technology (*Measurement of Enzyme-Substrate Affinities*) is an elegant solution to this unmet need for label-free drug measurement. It works by using x-ray excitation and x-ray fluorescence of heavy atoms. Many drugs contain these heavy atoms that are fluorescent in the x-ray spectrum.

Label-free protein-drug interaction measurements provide a means to answer questions concerning protein target identification during drug discovery and development. Utilizing phenotypic cell-based assays is appealing from a drug discovery point of view. However, one drawback to this approach has been subsequent identification of the molecular target responsible for a particular compound's mechanism of action. Increasingly, regulatory agencies such as the FDA requires that the protein target be identified in order to grant a drug Investigational New Drug (IND) status, i.e. approval for human clinical trials. Traditionally this has involved chemical modification of the ligand with a fluorescent label. However, in many instances installation of the label results in diminished activity of the derivative compared to the parent molecule. MESA can allow label-free measurement of protein-drug interaction useful for target identification. This could unlock tremendous value by simplifying the process of target identification and would encourage increased utilization of cell-based assays in drug discovery.

Label-free measurement of protein-drug interaction has been a longstanding need in the pharmaceutical industry. The preliminary MESA data is promising, and if it can be implemented on an industrial scale, it could significantly affect the development of new lifesaving drugs.

Sincerely,

Gregory Cuny, Ph.D.
Director of Medicinal Chemistry
Laboratory for Drug Discovery in Neurodegeneration
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Harvard Medical School
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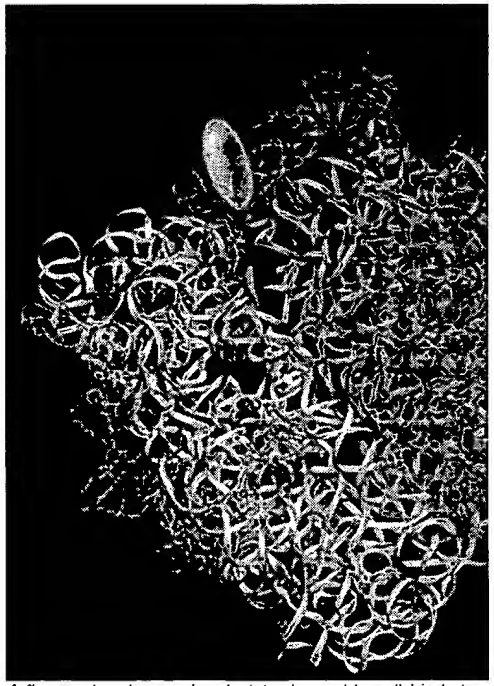
Ph: (617) 768-8640

MESA: Measuring Enzyme-Substrate Affinities

The MESA technology, developed in Chemistry Division, has the potential to revolutionize the way the pharmaceutical industry discovers and brings new drugs to market. This amazing technique has won a 2005 R&D 100 Award and is the core technology behind a new Los Alamos spin-off company, Caldera Pharmaceuticals Inc. Caldera will mature the technology and bring it to market.

MESA is a low-cost assay for detecting the binding of drugs to proteins (and other biomolecules and cell structures) without the biasing influence of added fluorescent molecular labels. The assay images drug-protein binding using atoms intrinsic to drug molecules themselves. Because of this label-free detection, MESA captures and quantitates all drug-protein binding, including bindings that are potentially therapeutic and those that are potentially toxic. This allows MESA measurements to generate a complete therapeutic index early in the drug-development process. Today's high drug-development failure rate—the primary cause of the high cost of new drugs—is driven by the inability to measure more than an infinitesimal number of protein-drug interactions. It is estimated that the cost for fully developing a new drug is well over a billion dollars. MESA's ability to measure a very large number of these interactions and its resulting early detection of toxicity could prevent the late stage clinical failures that consume up to 80% of pharmaceutical development costs.



A fluorescing drug molecule (glowing gold oval) binds to a protein (twisted-and-coiled thin teal "rope") within a "ribbon" representation of a bacterial ribosome, a frequent target for antibiotic drugs. This binding of the native drug to protein molecule would be unambiguously detected by MESA label-free measurement technology. The currently standard techniques, which rely on detecting a drug whose structure has been altered by an attached fluorescent label, might not detect the binding.

Applications

- Drug Development: Screens label-free drugs against the proteome in 24–72

hours, compared with extant technologies that test drug effects on less than 0.5% of the body's proteins.

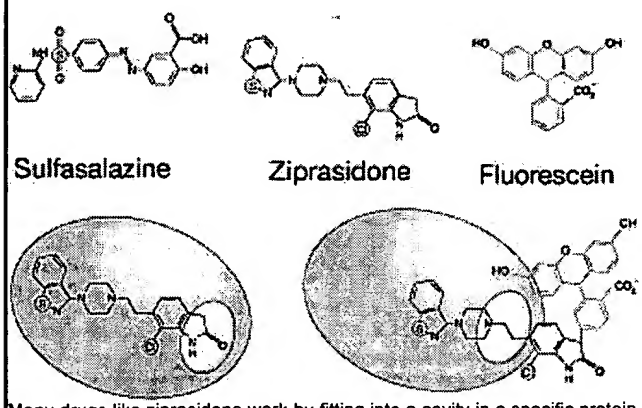
- Personalized Medicine: Allows individual patients to be screened for their likely response to drugs so that the right drug can be prescribed and adverse drug reactions can be reduced.
- Target Validation: Facilitates the identification of new protein targets for drug therapies, a necessity for developing cures for currently intractable or incurable diseases.

Postdoctoral researcher Edel Minogue binds proteins to a slide for analysis.

Benefits

- Fast: Measures drug selectivity for many proteins at throughputs comparable to best-in-class pharmaceutical industry standards for measuring much simpler single drug-protein affinity.
- Reveals therapeutic index: Identifies not only whether a drug will be effective, but also whether it will be safe.
- Inexpensive: Eliminates the need for fluorescent labeling, which consumes both time and money.
- Label-Free Accuracy: Provides far more accurate data than that obtained with fluorescently labeled molecules.

Comparison to state-of-the-art.



Many drugs like ziprasidone work by fitting into a cavity in a specific protein (light-blue spheroid). When drugs are labeled with compounds like fluorescein, they often no longer fit into a protein cavity (fluorescein-labeled ziprasidone, lower right) that would otherwise accommodate them (unlabeled ziprasidone, lower left); this yields false data and, therefore, compromises any data derived from the fluorescently labeled drug. By contrast, MESA uses the intrinsic features of many drugs to measure drug-protein binding.

Principal Developers: Benjamin Warner (C-SIC), George Havrilla (C-CSE), and Edel Minogue (C-SIC).

Graphics and text developed by IM-1. 7/05